VACCINATION AGAINST YELLOW FEVER WITH IMMUNE SERUM AND VIRUS FIXED FOR MICE

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The method here presented for vaccination against yellow fever was devised primarily to interrupt the long series of accidental infections of persons making laboratory investigations. In the $4\frac{1}{2}$ years which have elapsed since *rhesus* monkeys first came into use as experimental animals in yellow fever studies, there have been reported 32 infections with yellow fever in laboratories devoted to research in this disease, according to Berry and Kitchen (1), and five scientists have lost their lives.

Preliminary Experiments with Other Yellow Fever Vaccines

The results of experiments in this laboratory and the receipt of unfavorable reports from elsewhere have made us unwilling to recommend the immunization of persons against yellow fever with vaccines prepared by the chemical treatment of the virus-containing tissues of infected monkeys. Vaccines of this general type have been devised by Hindle (2), Aragão (3), Pettit and Stefanopoulo (4), and Monteiro (5). According to Chagas (6), irregular results followed the vaccination of 25,000 persons by the method of Aragão during the recent epidemic in Rio de Janeiro, and failures were also encountered in the experimental vaccination of monkeys. Okell (7) found that a vaccine of the same general type lost its immunizing power rapidly on storage, and this would explain the failures to immunize persons far from the place where the vaccine was prepared. Davis (8) made an experimental chloroform-treated tissue vaccine which was found, on laboratory test, to be sometimes infective and sometimes without immunizing effect. Burke and Davis (9) recorded the case of a person in Brazil who contracted a fatal infection with yellow fever 5 months after vaccination with monkey tissues treated with formalin and phenol. In March, 1931, a commission of the French Academy of Medicine (10) announced its conclusion that no method of vaccination against yellow fever had been sufficiently studied for use in practice.

Attenuation of Virus in Monkey Serum.—In this laboratory vaccination experiments with attenuated virus were carried out in 1929 and 1930 with the collabora-

tion of N. Paul Hudson. The virus in highly infectious monkey serum was progressively attenuated by exposure (*a*) to a temperature of 37°C., (*b*) to the effects of added formaldehyde (0.05, 0.1, or 0.2 per cent) or tricresol (0.3 per cent) at room temperature, or (*c*) to the effects of 0.3 per cent tricresol at a temperature of 37°C. or 0.5°C. Portions of the serum under treatment were withdrawn at intervals for testing. Some of these portions were injected at once subcutaneously into monkeys. Others were dried in the frozen state in test-tubes or ampoules by methods described in a previous publication (11), and stored for a week or more before injection into monkeys. Later the monkeys were given test inoculations of virulent yellow fever virus of the Asibi strain. The periods of exposure of the vaccine to the attenuating agents varied in the different experiments, but commonly ranged from an hour or less to several days. As a rule the vaccines exposed for the shortest periods to the attenuating agents produced yellow fever in the monkeys inoculated and the specimens long exposed failed to immunize. Between these extremes, there were found in some cases exposures which attenuated the virus to such an extent that the vaccinated animals showed no fever but acquired an active immunity. This zone in the series of exposure times was inconstant and narrow.

The irregular results convinced us that much further research would be necessary if a safe and dependable vaccine for human beings was to be prepared by the chemical treatment of virulent strains of yellow fever virus.

Simultaneous Injection of Virus and Immune Serum.—The practice of injecting virus and immune serum at the same time but in different places has been followed in vaccinating swine against hog cholera and cattle against rinderpest, and in immunizing against other diseases of animals. It is possible to immunize against at least one of these diseases, hog cholera, when the virus and serum have been mixed before injection, for Duval (12) has shown that the dried virus and the dried immune serum may be mixed, stored, and used together without the loss of antigenic power.

Many who, like ourselves, have had occasion to make protection tests of serum, using monkeys, have observed the presence of active immunity in those animals which have been protected against an injection of yellow fever virus by a simultaneous or previous injection of immune serum. This observation (Theiler and Sellards (13), Findlay and Hindle (14), Aragão (15)) has suggested the possibility of using the same general method in vaccinating man.

In one of our early experiments a solid active immunity was produced by mixing yellow fever virus of the potent Asibi strain in monkey serum, dried under vacuum in the frozen state and redissolved, with similarly dried immune monkey serum, and injecting the mixture subcutaneously into monkeys. The immunity was acquired in the absence of fever. Although the serum and virus were in contact for 30 to 40 minutes at room temperature, thorough immunization was accomplished when the amount of immune serum in the mixture was 5 or 10 times the minimum necessary to protect against the virus.

It seemed to us that it would be a valuable safeguard if the living yellow fever virus selected for use with immune serum in vaccinating human beings were of

lower virulence for man than the strains now being carried in monkeys. In vaccination against hog cholera, according to information given us by Dr. R. E. Shope, on rare occasions there occur unexplained failures of the immune serum to protect against the virus and as a result the vaccinated animals die of the disease.

Attenuation of Virus in Mouse-Brain Tissue.—When Theiler (16) showed that yellow fever virus which had been adapted to mice had lost much of its virulence for monkeys, the hope was raised that the untreated virus in mouse-brain tissue, after enough passages in mice, would lose its virulence for man and would be safe to use as a vaccine. At the time of our experiments the French strain of yellow fever virus had been through over 100 successive passages in mice and was nevertheless still able to produce fever in monkeys, although it had apparently long lost its power to kill them. Among 9 *rhesus* monkeys which were inoculated with this strain, after 102 to 120 passages through mice, and were afterward given test inoculations with virus from monkey source, 8 developed fever and all 9 were subsequently found to be immune. Attempts were made to transfer the infection from 4 of these animals to normal monkeys by inoculation with blood taken at the beginning of fever, but none of the animals receiving the blood developed fever; half of them were afterward shown to be susceptible, and the others immune, to yellow fever virus from monkey source (Asibi strain). Two control monkeys inoculated with normal mouse-brain tissue remained free from fever.

The gradual diminution of virulence for monkeys as yellow fever virus is passed through mice is illustrated by our experience with the Asibi strain. A monkey inoculated with virus of the 5th passage in mice died of yellow fever. Of 2 monkeys inoculated with virus of the 10th passage, 1 died of yellow fever and the other had fever followed by immunity. One which received the 11th-passage virus died of yellow fever. Monkeys inoculated with virus of the 15th, 20th, and 25th passages developed fever and were immunized. The French strain in the 176th passage in mice still produced definite attacks of fever in monkeys.

There is other evidence of the persistence of a degree of virulence for monkeys and man in yellow fever virus after many passages through mice. Sellards (17) has shown that such virus will cause encephalitis in monkeys if injected intracerebrally. Moreover, there are on record 3 mild cases of yellow fever contracted by persons through contact with experimentally infected mice or their tissues (1). Yellow fever virus after 100 or more passages in mice is probably of low virulence for man, but still capable of causing mild attacks of yellow fever. If used in vaccinating persons, it seems advisable, therefore, that the virus be attenuated or given with a potent immune serum.

Two methods of attenuating the virus in mouse brain were tried, those of Semple (18) and Alivisatos (19) for preparing antirabic vaccine. Modifications were necessary, and in some instances the vaccine was dried in the frozen state and stored before use. The vaccine of the Semple type, as prepared and administered to monkeys, was apparently devoid of immunizing power. That of the Alivisatos type produced fever and immunity in monkeys when the brain tissue had been exposed to ether at 0.5°C. for 6 or 24 hours, but was inert when the exposure had been for 48 or 72 hours.

Vaccination with Living Yellow Fever Virus Fixed for Mice and Immune Human Serum

The preliminary experiments with various ways of vaccinating against yellow fever seemed to show that the most dependable and effective of the methods tried was the injection of living yellow fever virus with a simultaneous or preceding injection of potent immune serum. Moreover, it appeared that yellow fever virus, after many passages through mice, had lost most, but not all, of its virulence for monkeys, and probably for man, although retaining its power to immunize. It was proposed, therefore, to test in monkeys the safety and the immunizing power of a vaccine composed of living virus fixed for mice and human immune serum, and, if these tests gave satisfactory results, to commence immunizing persons exposed to yellow fever.

Preparation and Administration of the Vaccine

The vaccine used throughout the experiments and in vaccinating people consisted of two parts, (*a*) the virus in mouse-brain tissue suspended in a portion of the immune serum or in normal serum, and (*b*) immune human serum for separate injection. In many experiments, however, both parts were combined. The description which follows gives the technic used in making most of the lots of the vaccine employed in immunizing persons, and at the end there is a statement of ways in which the preparation of the first vaccines, Vaccines A, B, 1, and 2, and the last, Vaccine 6, differed from the description. The essentials of the method of preparation of each vaccine used are given in Table I.

The Component Containing Virus.—The virus used in the preparation of the vaccines was of the French strain, 105th to 176th passages in mice. Only persons immunized against yellow fever by illness or vaccination were allowed to work with the virus. A 10 per cent suspension of the infectious mouse-brain tissue in immune serum was centrifuged for ½ hour at about 3,000 R.P.M. The supernatant fluid was then passed through a Seitz filter. As tests of sterility sets of aerobic and anaerobic cultures in slightly alkaline meat infusion peptone broth were made before and after filtration. The filtrate was placed in amounts of 1 cc. in sterile, plugged test-tubes and was dried in the frozen state under vacuum. The tubes were then sealed in the blast lamp and stored in the refrigerator.

Some of the vaccine, redissolved in distilled water, was injected intracerebrally into 6 susceptible white mice in amounts of 0.03 cc. and intraperitoneally into 4 mice in amounts of 0.2 cc. In all cases some of the mice given intracerebral injections died of yellow fever encephalitis, showing that there was living virus in the preparation. The mice receiving intraperitoneal injections remained well. At least 1 monkey was vaccinated with each lot of the material together with the required amount of immune serum and was tested later for immunity.

The Immune Serum.—The immune sera used for the separate injections at the time of vaccination, and also in suspending the virus in the preparation of the vaccine, were obtained from 6 members of the laboratory staff who had had yellow fever as the result of accidental laboratory infections (1), and from 3 other members who had been immunized by vaccination during these studies. Blood was withdrawn, defibrinated, and cleared of cells by centrifuging. Aerobic and anaerobic

TABLE I

Method of Preparation of the Vaccines Used

Designation of vaccine	Strain of virus used and No. of passages in mice	Human serum in which virus was suspended	Percentage of virus* in suspension	Filter used	Fresh or dried	Mortality ratio in mice†
A	French—105	No. 3, immune	10	Not filtered	Fresh‡ Dried	9/12
B	French—106	No. 3, immune	10	Not filtered	Dried	6/11
1	French—107, 116	No. 1, immune	1, 2.5, 5	Not filtered	Dried	7/18§
2	French—117	No. 2, immune	10	Berkefeld N	Dried	3/12
3	French—146	Pool A, immune	10	Seitz‖	Dried	9/11¶
4	French—168	No. 4, immune	10	Seitz	Dried	3/4
5	French—174	No. 5, immune	10	Seitz	Dried	12/12
6	French—176	No. 6, normal	10	Seitz	Dried	6/6

* Fresh mouse-brain tissue containing yellow fever virus.

† Mice were inoculated intracerebrally. The ratio shows the proportion of mice dying from 4 to 10 days after inoculation.

‡ When 3 weekly injections of this fluid vaccine were given, the 2nd and 3rd doses were freshly prepared by the same method but with new lots of virus. This vaccine was fresh in Experiment I and dried in Experiment IV (Table II).

§ Mortality ratio for 1 per cent virus, 2/6; for 2.5 per cent, 1/6; for 5 per cent, 4/6.

‖ Part through Berkefeld N, part through Seitz.

¶ The brain of 1 mouse was examined microscopically, and lesions of yellow fever encephalitis were found.

cultures in broth were made as tests of sterility. To the serum for use in the separate injections was added 0.2 per cent of tricresol in ether, by the method of Krumwiede and Banzhaf (20). Each serum was tested for potency by injecting it subcutaneously into *rhesus* monkeys in amounts of 0.3 cc. or more per kilo of body weight and thereafter injecting 0.4 cc. of monkey blood containing yellow fever virus of the Asibi strain, virulent for monkeys, under the skin in another place. Amounts of serum which protected the animals against yellow fever were considered suitable for use in the same dosage per kilo in vaccinating man.

Serum 1 consisted of several lots from W. A. S., who had been infected from monkey source. This serum had a titre of 1/128 when tested in mice by the intraperitoneal protection test (21). Serum 2 (titre, 1/256) from M. T. and Serum 3 (titre, 1/128) from S. F. K. came from persons who had been infected with virus from mice. The donor (K. S.) of Serum 4 (titre, 1/256) had been infected with virus from monkeys. L. M. M., the source of Serum 5 (titre, 1/128), had been immunized by vaccination. Serum 6 was normal serum without protective power. The blood of each donor had been found to give a negative Wassermann reaction.

Serum Pool A (from W. L., K. S., and T. N.), Pools 1 and 2 (W. A. S., S. F. K., M. T., T. N., and K. S.), and Pool 3 (W. A. S., S. F. K., M. T., and T. N.) were made up of sera from persons who had experienced attacks of yellow fever. Pool 2, in the amount of 0.3 cc. per kilo of body weight, protected a monkey against the Asibi strain. Pool 3 did not protect either of 2 monkeys when injected in the amount of 0.4 cc. per kilo of body weight. It protected when used in the amount of 0.5 cc. Pool 4 (titre, 1/256) was a mixture of the sera of 2 vaccinated persons, M. H. and L. B. T. When injected 6 hours before the virus, this serum protected only 1 of 2 monkeys in a dosage of 0.3 or 0.4 cc. per kilo, but it gave protection in the dosage of 0.6 or 0.8 cc. per kilo.

Deviations from the Technic Described.—In preparing Vaccines A, B, and 1, the suspension of brain substance in immune serum was neither centrifuged nor filtered. Vaccine 1 was prepared in 3 strengths containing 1, 2.5, and 5 per cent of brain tissue, respectively. Vaccine 2 was filtered successively through Berkefeld cylinders V and N, instead of through the Seitz filter. In preparing Vaccine 6, the virus-containing mouse-brain tissue was suspended in normal human serum instead of in immune serum, and the vaccine was dried in ampoules in 0.5 cc. amounts instead of in test-tubes in amounts of 1.0 cc.

Administration of the Vaccine.—In the vaccination experiments in which monkeys were used, all the serum and the virus were given as a mixture except in a few instances, in Experiments VIII and IX, in which supplementary serum was given immediately before the injection of the component containing the virus. In vaccinating persons, on account of the large volume of serum required, the usual method was to inject immune serum subcutaneously in 2 or 3 places on the abdomen, and to inject the virus-containing component of the vaccine, redissolved in sterile distilled water, subcutaneously at another point on the abdomen. The actual time interval between injections of serum and virus component was less than 5 minutes in Cases 4 and 9 to 12 (Table IV); about 30 minutes in Cases 1 to 3 and 5 to 8; and 6 hours in Cases 13 to 16.

Tests for Hypersensitivity to Mouse-Brain Tissue.—In 14 cases, all except Cases 4 and 16, a preliminary intradermal injection of 0.1 cc. of a suspension of mouse brain in human serum was given ½ hour or more before the injection of the virus component of the vaccine for the purpose of detecting any unusual sensitivity to mouse-brain tissue. 1 per cent suspensions, especially if filtered, produced only slight transient reactions. In the light of this experience and the observation that severe immediate reactions are seldom if ever encountered after the first injections

of the spinal cord or brain tissue of rabbits in antirabic treatment it was decided that the preliminary intradermal test was unnecessary.

Vaccination of Monkeys

Nine experiments in the vaccination of *rhesus* monkeys with mixtures of virus fixed for mice and immune human serum were performed with the vaccine preparations and sera already described. The results are brought together in Tables II and III.

Most of the animals showed no reaction to the vaccination during an observation period of at least 30 days. Some had rises of temperature to 40°C. or above, which are recorded in Tables II and III as fever. The temperatures of the monkeys were taken twice each day, and the tissues of animals that died were examined histologically. Blood drawn long enough after vaccination to rule out passive immunity was tested in monkeys or mice, or in both, for its protective power against yellow fever virus. Each vaccinated monkey was later given a test inoculation of virulent virus of Asibi strain from monkey source.

In Experiment IV (Table II) fresh immune serum (Serum 3) was added to the redissolved vaccine to give the required percentage of virus, and in Experiment VII, Vaccine 2 was similarly diluted with Serum 1. In experiments VIII and IX (Table III) supplementary serum was given in separate subcutaneous injections when the smaller quantities of virus were used.

Experiments by Bauer (22) enabled us to rule out passive immunity in all the experiments except No. VII. Monkeys which had received much larger amounts of human yellow fever immune serum in his experiments had lost their passive immunity within 14 days. Passive immunity could not have been present even in the first 8 days of Experiment VII, for in human vaccinations, to be discussed later, it was shown that amounts of serum, equal to those given to monkeys in these experiments, produced no passive immunity that could be detected a few hours after vaccination.

When the sera of vaccinated animals were tested in monkeys, the serum was injected intraperitoneally in the amount of 1.5 cc. per kilo in Experiments I and II, and 3 cc. per kilo in Experiments III and V. 6 hours later each of these monkeys and at least 1 control were given subcutaneous injections of 0.4 cc. of the Asibi strain of yellow fever virus in monkey blood.

In the tests of the serum in mice, and in all titrations, the technic of the intraperitoneal protection test of Sawyer and Lloyd (21) was followed.

The interval between vaccination and test inoculation with the Asibi strain was 40 days in Experiments I and II, 76 days in Experiments III and IV, 46 days in Experiment V, 73 days in Experiment VI, 47 days (Monkeys 29 and 31) and 92 days (Monkeys 30 and 32) in Experiment VII, 55 days in Experiment VIII, and 38 days in Experiment IX.

TABLE II

Results of Vaccination of Rhesus Monkeys with Yellow Fever Virus Fixed for Mice and Immune Serum

Experiment	No. of monkey	No. of vaccine	Virus content	Volume injected per kg. of body weight	No. of weekly injections	Reaction to vaccination	Results of test inoculation	Interval from 1st injection to bleeding	Tests of monkey serum	
									Protection test in monkeys. Result*	Protection test in mice. Result
			per cent	*cc.*				*days*		
I	1	A	10	0.1	1	Fever	No reaction	35	Partial protection	
	2	A	10	0.1	3	Fever	No reaction	35	Partial protection	
	3	A	10	1.0	1	No reaction	No reaction	35	No protection	
	4	A	10	1.0	3	No reaction	No reaction	35	No protection	
II	5	B	10	1.0	3	Fever	No reaction	40	Protection	
	6	B	10	1.0	3	Fever	Died; not yellow fever	40	No protection	
III	7	1	1	0.3	1	No reaction	No reaction	66	No protection	
	8	1	1	0.3	1	No reaction	No reaction	66		Protection
	9	1	1	0.3	3	No reaction	No reaction	66	Partial protection	No protection
	10	1	2.5	0.3	1	No reaction	No reaction	66		Inconclusive
	11	1	2.5	0.3	3	No reaction	No reaction	66		Inconclusive
	12	1	5	0.3	1	No reaction	No reaction	66	No protection	
	13	1	5	0.3	1	No reaction	No reaction	69		Inconclusive
	14	1	5	0.3	3	Fever†	No reaction	69	Protection	
IV	15	A	5	0.3	1	No reaction	No reaction	69		Protection
	16	A	5	0.3	3	No reaction	Died; colitis‡	69		Protection
	17	A	5	0.3	3	Fever	Died; colitis‡	69		Inconclusive
	18	A	1	0.3	1	No reaction	No reaction	69		Protection
	19	A	1	0.3	3	No reaction	No reaction	69		Protection
V	20	1	2.5	0.3	1	Fever	No reaction	40	Protection	Protection
	21	1	2.5	0.3	2§	No reaction	No reaction	40	Protection	Protection
	22	1	2.5	0.3	3§	No reaction	No reaction	40	Protection	Protection
VI	23	2	10	0.3	1	No reaction	Fever	73		Protection
	24	2	10	0.1	1	Fever	No reaction	73		Protection
	25	2	10	0.3	2	No reaction	No reaction	73		Inconclusive
	26	2	10	0.3	3	Fever	No reaction	73		Protection
Control	27	2a‖	10	0.3	1	No reaction	No reaction	73		Inconclusive
Control	28	2b¶	0	0.3	1	No reaction	Died; yellow fever			
VII	29	1	1	0.3	1	No reaction	No reaction	8		Protection
	30	1	1	0.3	1	Fever	Fever	8		Protection**
	31	2	1	0.3	1	No reaction	No reaction	8		Protection
	32	2	1	0.3	1	No reaction	No reaction	14		Protection††

* "Protection" signifies that the monkey was free of fever and survived; "partial protection," fever and recovery; "no protection," death from yellow fever.

† One rise of temperature a few hours after 2nd injection. No other fever.

‡ Died 23 days after vaccination. No evidence of yellow fever.

§ Second injection consisted of only 0.1 cc. of the serum-virus mixture per kilo of body

From the experiments in the vaccination of monkeys (Tables II and III), the following facts seemed to emerge.

1. The monkeys which received a dried mixture of 0.003 to 0.03 gm. of yellow fever virus fixed for mice and 0.3 cc. of immune human serum per kilo of body weight, with or without additional inoculations of virus and serum, were immunized against yellow fever, and most of them developed demonstrable antiviral substances in their blood.

TABLE III

Vaccination of Monkeys with a Constant Amount of Immune Serum and Varying Amounts of Virus

No. of experiment	No. of monkey	No. of vaccine	No. of serum pool	Immune serum per kg.	Virus, mouse-brain, per kg.	Reaction of monkey to vaccination	Result of test inoculation	Results of protection tests in mice: Days between vaccination and bleeding* 7	14	21	24	Titre of serum of monkey
				cc.	gm.							
VIII	33	3	1	0.3†	0.003†	None	Not done	±	+		+	
	34	3	1	0.3	0.003	None	Fever	−			+	24th day, 1/256
	35	3	1	0.3	0.001	Fever	No reaction	−	+		+	
	36	3	1	0.3	0.0003	None	No reaction	−			+	
IX	37	4	3	0.3‡	0.03	None	No reaction	±	+	+		28th day, 1/128
	38	4	3	0.3	0.003†	Fever	No reaction	+	+	+		28th day, 1/128
	39	4	3	0.3	0.0003	None	No reaction	±	+	+		28th day, 1/64
	40	4	3	0.3	0.00003	Fever	Fever	±	+			28th day, 1/64

* + signifies "protection;" ±, "inconclusive;" −, "no protection."

† The amount most commonly used in vaccinating persons.

‡ In this amount Serum Pool 3 was found to have too low a protective power against Asibi virus from monkey source for use in vaccinating persons.

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weight; 3rd injection, 0.1 cc. of 5 per cent fresh virus (mouse-brain) in normal human serum per kilo.

‖ Normal human serum used in place of immune serum.

¶ Immune serum without virus, to rule out persistence of passive immunity in tests of serum of vaccinated monkeys.

** Titre of serum by protection test in mice on 56th day after vaccination was 1/512, the highest dilution tested.

†† 8 days after vaccination the result was "no protection."

Of 23 such monkeys (Table II, Experiments III to VII, excluding Monkeys 24, 27, and 28), 18 had no reaction during the observation period of 30 days, and 5 had rises of temperature to 40°C. All were given test inoculations with virulent yellow fever virus of the Asibi strain. Two died of ulcerative colitis without any evidence of yellow fever. The other 21 survived. The sera of 7 of these 23 vaccinated monkeys were tested in monkeys for protective power: 4 protected completely, 1 permitted fever, and 2 gave no protection. The sera from 20 of the 23 vaccinated monkeys were tested in mice: 14 of the sera protected, 5 gave inconclusive results, and 1 gave no protection.

2. One injection of the vaccine was as effective in the production of immunity as 2 or 3 weekly injections (Table II).

3. The immunizing power of the vaccine did not vary significantly with its virus content, although the amount of the virus-containing brain tissue ranged from 0.00003 gm. per kilo of body weight to 0.03 gm., the largest amount being 1,000 times the smallest (Table III).

4. Antiviral substances, sufficient in amount to be detected by the protection test in mice, were sometimes present in the blood of monkeys as early as 8 days after vaccination (Table II, Experiment VII).

5. The few vaccinated monkeys whose serum had not acquired the power definitely to protect monkeys or mice against yellow fever virus were nevertheless resistant to test inoculation.

6. As symptoms following vaccination were absent or slight, this method of immunizing was safe for monkeys and probably would be harmless to persons.

Vaccination of Persons

Sixteen persons were vaccinated against yellow fever in this laboratory, the first on May 13, 1931, by the methods developed in the experiments with monkeys. In addition, 3 persons in Nigeria and Brazil have been immunized effectively with materials sent from this laboratory. The essential facts regarding the vaccinations performed by us are shown in Table IV.

The preparation of the vaccines has been described in the preceding text and Table I. In Cases 1 to 4, 11, and 12, the virus suspension had not been centrifuged or filtered. In Case 4 most of the immune serum (16 cc.) had been dried with the virus and the additional 5 cc. needed had been dried in the frozen state 2 months before. In the other cases the immune serum for separate injection had been tricresolized and stored as fluid.

Although the dried specimens of Vaccine 1 used in Cases 11 and 12 had been stored for nearly 8 months in the refrigerator, they still had high immunizing

TABLE IV

Results of Vaccination of Persons with Yellow Fever Virus Fixed for Mice and Immune Serum

No. of case	Person vaccinated	No. of vaccine	No. of serum	Amount of virus per kg. of body weight	Amount of immune serum per kg.	Tests of person's serum for protective power			
						In white mice		In *rhesus* monkeys	
						Interval from vaccination to		Interval from vaccination to	
						Last negative (−) or inconclusive (±) result*	1st positive	Last negative result†	1st positive‡
				gm.	*cc.*	*days*	*days*	*days*	*days*
1	D. B. W.	1	1	0.003	0.3	7 (±)	9	0	19
2	E. H.	1	1	0.003	0.3	8 (±)	14	0	23
3	L. B. T.	1	1	0.003	0.3	8 (±)	14	7	23
4	V. G.	1	1	0.003	0.3	8 (−)	12	0	15
5	T. P. H.	2	1	0.003	0.3	7 (−)	14	14	23
6	L. M. M.	2	1	0.003	0.3	7 (−)	14	−3	14
7	N. R.	2	1	0.003	0.3	7 (−)	14		14
8	P. J. C.	2	1	0.003	0.3	12 (±)	21		89
9	G. W. M.	3	1	0.003	0.3	6 (±)	9	−6	89
10	J. P. C.	3	Pool 1	0.003	0.3	−3 (−)	7		84
11	J. S. C.	1	Pool 2	0.0015	0.3	7 (±)	10	−4§	34
12	M. H.	1	Pool 1	0.001	0.3	0 (−)	7	0§	36
13	J. H. B.	4	1	0.003	0.3‖	7 (−)	11		
14	C. R. E.	4	1	0.003	0.3‖	7 (±)	11		
15	H. E. H.	4	1	0.003	0.35‖	10 (−)	14		
16	J. H. P.	6	Pool 4	0.0008	0.6‖	17 (±)	21		

* When inconclusive results are shown, there was an earlier negative result.

† Two monkeys were used in Case 5, in other cases only 1. Numbers preceded by minus signs show the number of days before vaccination.

‡ Two test monkeys were used in each case. Control monkeys receiving virus and normal serum died of yellow fever.

§ In an additional test 21 days after vaccination 1 monkey survived and 1 died of yellow fever.

‖ Serum given 6 hours before the virus component.

power. This evidence and the observation (Table III) that even one one-hundredth of the usual amount of virus would produce strong immunity in monkeys, led us to fix the quantity of virus to be used in vaccinating human beings, irrespec-

tive of weight, beginning with Case 16, as that which is present in 0.5 cc. of a 10 per cent suspension of infective mouse brain in normal human serum after filtration through a Seitz disc and storage in the dried state in the refrigerator for not more than 6 months. A man weighing 70 kilos, for example, would now receive only 0.0007 gm. of mouse brain per kilo, minus the loss by filtration. While body weight would no longer determine the amount of virus, it would still decide the amount of immune serum.

Symptoms after Vaccination

The first person to be vaccinated was admitted to the Hospital of The Rockefeller Institute for Medical Research through the courtesy of Dr. Rufus Cole and Dr. T. M. Rivers, and was closely observed for us by Dr. G. P. Berry of the hospital staff. Six hours after vaccination there was decided tenderness in the abdominal wall where the unfiltered virus component of the vaccine had been injected, but none where serum alone had been given. The tenderness disappeared in 2 days. There were no subjective symptoms and no abnormalities of temperature, pulse, blood pressure, heart action as shown by electrocardiogram, or urine, during 10 days in the hospital, 2 before vaccination and 8 after.

The other persons vaccinated remained on duty in the laboratory. Outside the hospital the vaccinated persons took their own temperatures at intervals of 4 hours during the day for a period of 2 weeks and recorded any subjective symptoms. The symptoms following vaccination were few and may be briefly summarized in 4 groups as follows:

1. Early Reactions to the Injected Mouse-Brain Tissue.—These symptoms were most in evidence during the evening following vaccination, and they disappeared in from 1 to 3 days. They were (*a*) tenderness at the site of injection of the virus suspension, sometimes with slight redness and swelling, and (*b*) rises of temperature, with headache or dull pain in the back or legs in 3 instances. Tenderness was present in all cases except Nos. 8, 12, and 16, and was severe in Cases 4 and 6. In 9 of the 16 cases the temperature rose above normal, but in only 1 did it go above 37.4°C. Except in Case 6, these early symptoms were slight or absent when the injected virus suspension had been filtered. Leucocytosis was commonly present.

2. Symptoms Suggestive of Late Reactions to the Injected Mouse-Brain Tissue.—(*a*) 7 days after vaccination, in Case 2, a lymph node near one of the sites of injection of unfiltered virus suspension became slightly enlarged and tender. On the

following day the skin at the site of the intradermal injection of the same suspension became reddened and elevated in an area 1.5 by 2 cm. and was surrounded at a distance of a few centimeters by 6 small papules (2 by 3 cm.) topped by vesicles 1 mm. in diameter. 1 day later similar papules appeared here and there on the arms and legs. The temperature was 37.2°C. on the 8th and 9th days.

(*b*) 7 days after vaccination, in Case 12, one of the sites of injection of unfiltered virus suspension became red and slightly swollen, and the temperature rose to 37.2°C. The swelling measured 4 by 5 cm. It was not tender, but there was itching. The swelling disappeared in 3 days.

(*c*) A mild attack of arthritis began 14 days after vaccination in Case 10. The fever lasted 3 days. There was tenderness for 4 days in several joints, including the knees, the hips, an elbow, and the temporomaxillary articulation. Blood taken on the 2nd day of fever and injected into a *rhesus* monkey did not immunize the animal against a later injection of yellow fever virus. The vaccinated person had had a similar attack of arthritis 11 months earlier, and he has had another such attack along with a "cold" 5½ months after vaccination.

(*d*) 13 days after vaccination, in Case 15, there was slight soreness of an ankle on motion. (A similar condition in the right elbow was reported as being present in the case of H. W. B. from 6 to 13 days after vaccination in Nigeria with Vaccine 3 and Serum Pool 2.)

In the 2 instances in which there were late reactions at sites of injection of the virus suspension, the injected material had not been centrifuged or filtered and therefore contained tissue particles. It seems probable that these inflammations of skin and lymph gland and also the arthritis were late reactions, of the serum disease type, to the injected foreign tissue. According to Boots and Swift (23), serum sickness is manifested during the 2nd and 3rd weeks following serum treatment by fever, lymph node enlargement, urticaria, transitory leucopenia followed by leucocytosis, and less frequently by joint pain and tenderness.

3. Symptoms Probably Having No Relation to the Vaccination.—In this group are included (*a*) rises of temperature following the extraction of infected teeth in Cases 1 and 8, and (*b*) symptoms of "colds" in Cases 6, 11, 14, and 16.

4. Symptoms Possibly Related to the Virus Injected.—In this group are included symptoms which may have been due to an immunizing yellow fever infection held in check by immune serum, although some of them may have been late reactions to foreign tissue, like the symptoms mentioned under "2." The leucopenia discussed in the following section should be classed with this group. In Case 1, on the 11th day after vaccination there was chilliness, and on the 12th, a rise of temperature to 37.4°C. with headache. In Case 6 there were rises of temperature to between 37.2° and 37.9°C. almost daily from the 8th to the 13th days. In Case 7 the temperature rose to 37.3°C. on the 8th day and headache and backache were present. In Case 9 there was a rise to 37.2°C. on the 10th day. In Case 15 the temperature was 37.2°C. on the 6th day after vaccination.

The Leucocytic Reaction to Vaccination

To secure evidence on the nature of the immunizing reaction to vaccination and make possible comparison of the blood cell changes with those observed by Berry and Kitchen (1) in attacks of yellow fever, the following study of the blood of the vaccinated persons was carried out.

Total leucocyte counts were made each forenoon, except for a few omissions, beginning 2 or 3 days before vaccination. The results are presented in Table V. The immediate reaction of the white blood cells, observed 4 to 6 hours after the injection of the virus suspension, was in most instances an abrupt rise. This was followed by a decrease, noticed the next morning. Over the ensuing several days there occurred an additional and more gradual fall, interrupted occasionally by irregular rises, until the count was in most cases below normal. Of 16 persons vaccinated, 14 showed some degree of leucopenia between 7 and 10 days after vaccination. In 5 cases the low point was below 4,000; in 5, between 4,000 and 4,999; in 4, between 5,000 and 6,000. In the remaining 2 cases it was over 6,000, but in 1 of them several counts were omitted.

Differential white cell counts were made on the first 9 persons vaccinated. They revealed no constant deviation from the normal in the ratios. In 5 of the 9 cases there was an increase of neutrophiles a few hours after vaccination, at the height of the immediate reaction to the foreign tissue. The leucopenia was due chiefly to a decrease in the number of neutrophiles. The changes in the numbers of lymphocytes and monocytes were not sufficiently consistent to be significant.

A degree of leucopenia was observed which was in general less than that seen in yellow fever, but the time of occurrence was consistent with that in yellow fever if allowance be made for an incubation period. The time from vaccination to leucopenia would seem to correspond approximately to the time between onset of illness and leucopenia, plus the incubation period.

As leucopenia may occur in serum sickness (23), we considered the possibility that the low white cell counts observed after vaccination might be late reactions to the injection of foreign tissue rather than effects of the virus. Against this interpretation is the observation that definite leucopenia was not observed in any of 3 persons immune to yellow fever by reason of attacks of the disease, who were inoculated with mouse-brain tissue as controls, although they showed the usual immediate rise after the injection of the foreign tissue (Table V). Two of these persons (S. F. K. and M. T.) received normal tissue and 1 (W. A. S.) infective tissue. The amount of brain tissue injected subcutaneously into W. A. S. and S. F. K. was approximately the same as that given in Case 11 (0.0015 gm. per kilo), and the suspension was in immune human serum and was unfiltered. To M. T. was given a filtered suspension in normal human serum containing 0.003 gm. of virus per kilo minus the loss by filtration. The small number of these controls still left

TABLE V

Total Leucocyte Counts of 16 Persons Vaccinated against Yellow Fever and of 3 Persons Given Control Injections of Mouse-Brain Tissue

Case No.	Person	Days after vaccination or injection of mouse-brain tissue																		
		−4	−3	−2	−1	0*	0†	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	D. B. W.			10,100	9,800	10,000	7,200	9,500	7,800	9,400	9,850	10,800	9,300	8,400	7,100	5,300‡	8,100	7,300	9,100	10,000	6,500
2	E. H.			4,400	6,500	4,425	7,850	4,700	4,400	4,650	4,050	4,050	4,275	4,000	3,500	3,950	2,800	3,950	5,750	5,125	5,350
3	L. B. T.			8,550	8,000	7,450	12,100	7,950	6,350	7,600	7,500	7,400	6,175	4,600	3,400	3,950	6,200	8,700		5,675	5,550
4	V. G.			7,200	8,800	7,100	7,300	4,400	6,300	12,000	8,600	4,850	9,925	6,050	3,450	3,800	4,500	3,750	5,100	5,400	4,000
5	T. P. H.		10,300	6,000		7,800	11,700	8,475	5,000	6,050	6,800	6,200		5,100	5,900	6,250	4,450	8,250	7,000	7,400	
6	L. M. M.	7,775	7,400			8,200	11,100	8,100	7,100	7,800	7,150	6,250	8,200	5,950	5,100	4,300	7,000				7,500
7	N. R.		7,100	10,800		9,400	11,700	8,850	9,900	8,800	9,500	8,200		5,000	7,200	5,450	6,150	5,800	7,100		11,250
8	P. J. C.		9,800	8,700		8,100	9,200	10,700	7,800	6,700	6,200	7,800		4,400	7,400	8,500	7,200	6,900	6,200		
9	G. W. M.			10,350	8,400	6,000		9,700	6,800	5,750	7,200	4,700	5,150	5,950	3,050	4,650		7,350			
10	J. P. C.		6,400	7,100		4,750		8,400	10,000	7,750	9,450			6,800			7,150				
11	J. S. C.					6,300	10,400	7,700	8,400	7,200	6,450	6,600	7,250	4,650	5,600	7,350	8,450	7,300	9,150	9,300	8,100
12	M. H.	8,300	7,800	7,400	7,600	6,650	14,400	9,400	8,150	7,800	6,250	6,100		6,000	3,500	4,800	6,200		6,850	7,200	
13	J. H. B.					7,400		9,800	7,950	9,000			7,400	6,750	5,200	6,200	7,050	9,800		10,300	
14	C. R. E.					9,600		11,200	10,300	10,100			7,800	7,200	10,600	7,600	6,600	9,100		8,400	
15	H. E. H.				7,000	6,800	9,100	7,100		4,500	5,600		6,300	5,300	6,800	6,250	5,200	5,350			
16	J. H. P.	8,600	9,400	9,800	8,600	9,600	7,800	7,000	5,000	5,200	5,600	6,200	6,600	6,800	4,800	7,000		6,200	6,200	5,800	7,400
Control	W. A. S.			4,700		6,000	11,025	6,700	5,350		5,650	6,500	6,500	6,250	6,250	5,300		5,350			
Control	M. T.				8,300	9,600	12,300	9,300	9,350	14,800	9,200		8,300	10,650	9,200		9,200				
Control	S. F. K.					8,950	11,000	7,550	7,600	7,000	9,400		7,400	7,100	7,200	7,300	10,050				

* Counts in this column were made on the morning of, and preceding, the vaccination.

† Counts in this column were done from 4 to 6 hours following vaccination, except in Case 15 in which the interval was 1 hour.

‡ The lowest leucocyte count in each case is underlined.

some doubt as to the significance of the leucopenia, and further evidence was therefore obtained by making total white cell counts on monkeys.

Four monkeys which received an amount of filtered virus-containing mouse brain per kilo of weight equal to that most commonly used in vaccinating persons, showed a definite leucopenia with low points from 3 to 6 days after the injections. Two other monkeys given normal mouse brain and 1 control animal which was not inoculated, showed irregular fluctuations in white cell counts, but no definite leucopenia.

The evidence suggests strongly that the leucopenia observed after vaccination is a reaction to the virus rather than to the mouse-brain tissue.

The Immunity Produced by Vaccination

The effectiveness of the vaccination of persons against yellow fever was measured by carrying out intraperitoneal protection tests with their serum in mice (21), and in all 19 cases, including those of the 3 persons vaccinated in Nigeria and Brazil with materials sent from this laboratory, a substantial active immunity was demonstrated within a month after vaccination. As is shown in Table IV the sera of the 16 persons vaccinated in this laboratory acquired power to protect mice against yellow fever virus in from 7 to 21 days after vaccination. The serum of each had failed to protect before vaccination.

As the active immunity had been produced by injections of virus fixed for mice, it was important to find out whether the sera of the vaccinated persons would protect also against strains of yellow fever virus other than those adapted to these animals. The sera of the first 12 persons vaccinated were therefore tested in *rhesus* monkeys against the Asibi strain of yellow fever virus from monkey source, a strain known to be virulent for monkey and man. In every case the serum protected each of 2 monkeys against death, and in most cases against fever also, when injected intraperitoneally in the amount of 1.5 cc. per kilo of body weight.

That passive immunity played no part in the observed protection by the sera of vaccinated persons was proved by testing the sera of the first 3 persons, by the protection test in mice, approximately 2, 6, and 24 hours after vaccination. Two negative results and 1 inconclusive result were obtained for each time interval.

Moreover, in most cases, including Cases 15 and 16 in which the serum was given in larger dosage, there was evidence against the presence of passive immunity in the negative and inconclusive results between the time of vaccination and the first positive.

The rise of the protective power of the sera of vaccinated persons and the subsequent fluctuations were followed by titration in mice.

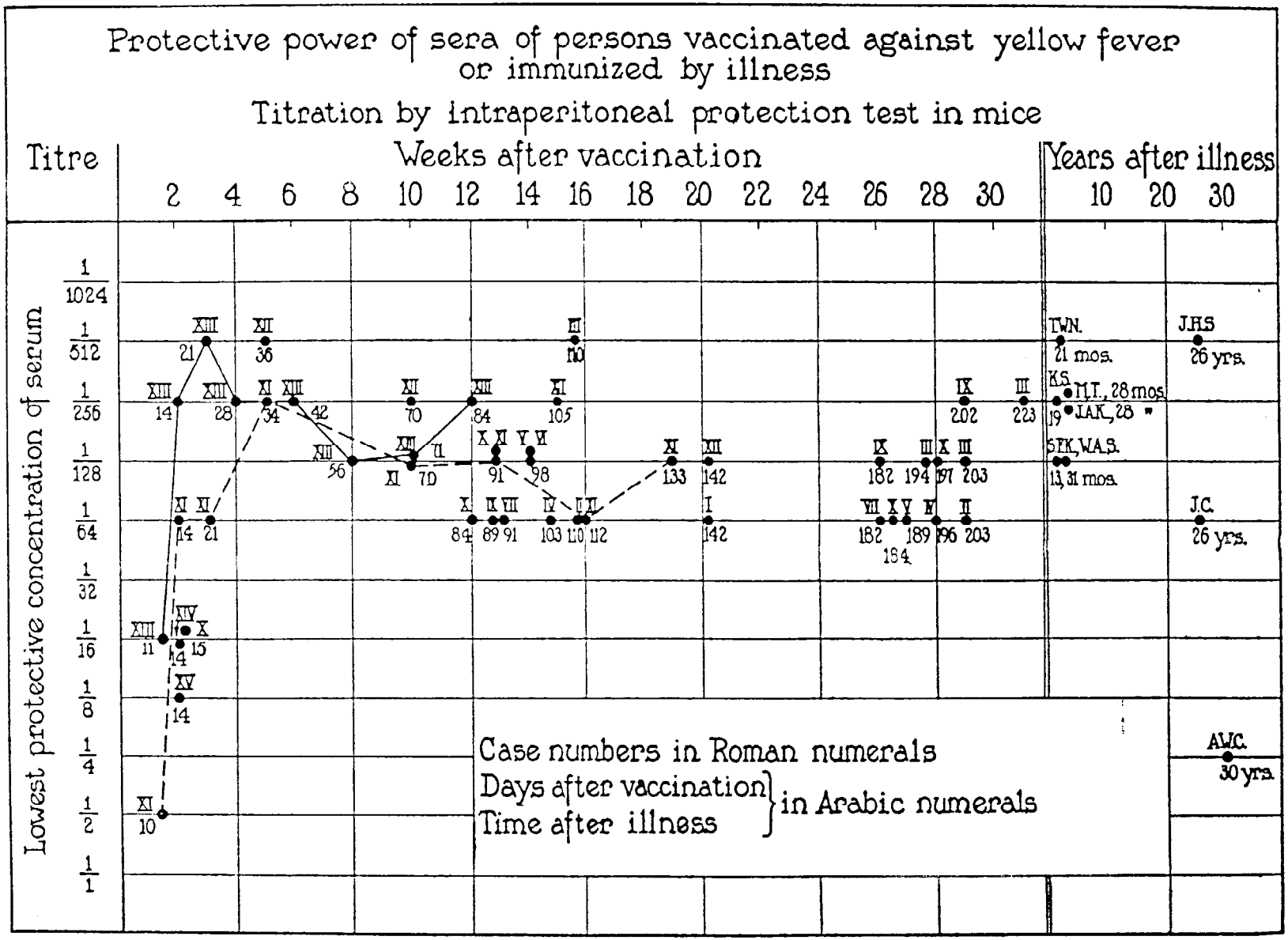

FIG. 1. Titres of human sera taken at various intervals after vaccination or after attacks of yellow fever.

The results of the titrations are brought together in Fig. 1. We are indebted to Dr. T. P. Hughes and Dr. M. Hoskins for making the titrations, and to Dr. J. S. Cunningham and Dr. J. H. Bauer for assistance in preparing the many serial dilutions required.

For comparison, the titres of sera from persons who have passed through attacks of yellow fever are shown in Fig. 1. The 6 recent cases are mentioned in the publication by Berry and Kitchen (1). For the sera of the 3 early cases we are indebted to Colonel J. F. Siler, Chief Health Officer of the Canal Zone, and Dr. L. B. Bates, Chief of the Board of Health Laboratory, Canal Zone. A. W. C. had yellow fever in 1901 in Cuba in one of the experiments of the Army Commission under Walter Reed. J. H. S. and J. C. had their attacks in the Canal Zone in 1905.

From Fig. 1 and Table IV it appears that, as a rule, the protective antibodies became demonstrable by tests in mice during the 2nd week after vaccination and increased to a maximum in the following 2 or 3 weeks. After that, they remained at about the same level for at least 6 months, most of the titres lying between 1/64 and 1/256.[1] The average titre was a little lower than the average for the sera from recent cases of yellow fever, but the difference may not have been significant in view of the small number of these recovered cases. Of the sera from persons who had had yellow fever from 26 to 30 years ago, one had a titre at the top of the range for vaccinated persons, another's titre was at the bottom of this range, and the titre of the third was much lower.

The Infectivity of the Blood after Vaccination

If there were yellow fever virus in the blood of vaccinated persons during the process of immunization and the mosquito vector were present, there would be risk of transferring the infection and starting an epidemic. This risk would be serious if the virus were from monkey source, but would seem to be remote if it were the neurotropic strain fixed for mice. Earlier in this paper we have mentioned the difficulty

[1] Since the completion of this article titrations of the sera of 3 of the vaccinated persons have been made from 5 to 10 months after vaccination. In Case 6 a titre of 1/8 was obtained 27 weeks after vaccination. In Cases 5 and 11 uncompleted titrations show that the titres will be below 1/32.

of carrying infection with the fixed virus from one monkey to another by the transfer of blood. It is, however, theoretically possible that the fundamental changes which have taken place in the virus during its adaptation to mice could be reversed under unusual conditions, and it cannot be too strongly urged that the living yellow fever vaccine virus should not be sent into regions in which yellow fever is absent but its vector is present, or introduced into other countries without the sanction of the government health authorities.

To determine whether virus was present in the blood of vaccinated persons several tests were made. Dr. Max Theiler cooperated by making many of the tests in mice for infectivity.

The intervals between vaccination and the taking of blood were 2, 6, 24, and 48 hours in Case 1; 2, 5, and 24 hours in Case 2; 2, 5, and 24 hours, and 7 days in Case 3; 3, 4, 6, and 7 days in Case 15; and 2 and 3 days in Case 16. Each specimen was defibrinated and injected intracerebrally into 12 or more highly susceptible mice. In most cases the blood was centrifuged and the serum injected into one set of 12 mice and the washed corpuscles into another. In Case 16 only the serum was used. In no case was virus recovered. There were a few deaths which might have been due to the virus, but in those instances in which subinoculation was possible or histological examinations of the brain were made, the diagnosis of yellow fever was ruled out.

In certain cases some of the blood was injected also into *rhesus* monkeys: 10 cc. of the washed blood cells of the 6-hour specimen in Case 1, and 3.5 cc. of the defibrinated 4-day specimen and 5 cc. of the washed blood cells of the 7-day specimen in Case 15. Each of the 3 monkeys succumbed later to test inoculation. Evidently they had not been immunized and the virus had not been present in the injected blood.

It seemed clear that in every instance the virus was absent from the blood of the vaccinated persons tested, or at least was very small in amount. On account of the remaining uncertainty the following experiments in monkeys were undertaken.

(*a*) A *rhesus* monkey was inoculated subcutaneously with 0.3 cc. of Vaccine 4 per kilo of body weight. It received in this way, on the basis of body weight, the usual amount of immune serum used in vaccinating persons, and 10 times the usual amount of virus. Blood was taken for test 2 hours and 20 hours later, and each sample was injected intracerebrally into 24 mice, 12 of which received defibrinated blood and 12, serum. The results were like those obtained in the tests of human blood. Only a few mice died, and in 1 instance subinoculation excluded yellow fever as the cause of death.

(*b*) Two *rhesus* monkeys were inoculated with the usual amount of virus but only one-tenth the usual quantity of immune serum. The blood of one monkey was tested after 1, 3, 5, 7, and 9 days, and that of the other after 2, 4, 6, 8, and 10 days. In each test 12 mice were inoculated intracerebrally with serum from defibrinated blood. The specimens taken 2, 3, 4, and 5 days after inoculation of the monkeys caused heavy mortality in the mice, and the cause of death was shown to be yellow fever by subinoculation and histological examination. The virus was not recovered from the specimens taken after other periods.

(*c*) Of a group of 4 *rhesus* monkeys, 2 were given subcutaneously 0.5 cc. of immune human serum per kilo of body weight. 3 hours later all 4 monkeys were inoculated subcutaneously with 0.03 cc. of Vaccine 6 per kilo. In this vaccine they received the usual amount of virus, but no immune serum. Of the 2 monkeys which had received immune serum, one had no reaction and the other developed fever. From 1 to 7 days after vaccination each monkey was bled on alternate days, 1 of each pair on the even days and the other on the odd days. Twelve or more mice were inoculated intracerebrally with each blood specimen. The mice which had received blood taken 1, 2, and 3 days after inoculation from the monkeys which had not been given immune serum showed a high mortality, and the virus was recovered from each group of mice. The virus was not recovered from the blood of the monkey which had received immune serum and virus and had shown no rise of temperature, and there were very few suspicious deaths among the mice receiving this blood. The mice inoculated with blood from the monkey which had been given immune serum and virus but had nevertheless reacted by showing fever, also had a low mortality, but the virus was recovered from 1 mouse which had been given blood drawn 3 days after vaccination. It seems that the virus was present in very small amounts in the blood of the monkey which had not been completely protected by the immune serum. Daily counts of the leucocytes in the blood of each of these 4 monkeys were made. There was leucopenia in each case, with the lowest number of cells from 4 to 6 days after inoculation.

In these tests we were unable to recover the virus from the blood of persons or monkeys vaccinated with fixed virus and sufficient immune serum to prevent fever, but the virus was found to be abundant in the sera of monkeys given fixed virus and much less than the usual amount of immune serum. Virus was present in extremely small amount in the blood of 1 monkey which developed fever after receiving almost enough serum. It is concluded that virus may circulate in the blood after vaccination if the amount of immune serum injected is not adequate, and that this dangerous occurrence may be prevented by using immune serum in excess of minimal requirements.

The Nature of the Immunizing Reaction

In our experiments it was shown that yellow fever virus could be mixed with proportionately large quantities of immune serum and kept at room temperature during several hours of preparation without losing the power to produce a substantial active immunity after injection into monkey or man. On superficial consideration this experience would seem at variance with the frequent observations of others that mixtures of viruses and large amounts of immune serum are without immunizing power.

J. H. Bauer (24), in an unpublished experiment cited with his permission, kept 5 per cent of yellow fever virus (infectious monkey serum) in immune human serum at 37°C. for 2 hours. He then injected the mixture into 10 monkeys in amounts ranging from 0.001 to 3.0 cc. There were no reactions, and all but 1 of the monkeys died later of yellow fever after test inoculation. The virus in the mixture had evidently been completely neutralized and had lost its immunizing power. In another experiment he showed that a mixture of 1 cc. of immune serum with 0.1 cc. of virus (infectious monkey serum), after exposure to room temperature in the African tropics for 45 minutes, would produce active immunity when injected into a monkey, but that a mixture of the same amount of serum and only 0.01 cc. of virus would produce no immunity.

To gain information about the extent to which neutralization might occur in the mixtures of virus and immune serum used by us in vaccination, the following experiments were performed.

Experiment A.—Two 10 per cent suspensions of the fixed yellow fever virus were made, one in normal human serum and the other in immune human serum. They were centrifuged and filtered through a Seitz disc. Each suspension was divided into two lots, one of which was kept at 28°C. and the other at 3°C. The mixtures kept at 28°C. were tested after 4, 24, and 96 hours, and those at 3°C. after 24 and 96 hours. In every test, a monkey was inoculated with 0.3 cc. of the suspension per kilo of body weight, and in all except the tests of the 4-hour specimens 0.03 cc. of the material was injected intracerebrally into each of 6 susceptible mice.

All the monkeys which received mixtures containing normal serum and the one given the 4-hour specimen containing immune serum became highly immune, for their blood serum developed a high titre for protective antibodies, ranging from 1/64 to 1/512. Moreover, all these monkeys later survived test inoculation, except 1 which died of other cause than yellow fever. Every mouse inoculated with the same mixtures died, presumably of yellow fever. The monkeys which received the mixture containing immune serum, after it had been exposed to tem-

peratures of 28°C. or 3°C. for 24 hours or longer were not immunized, for all died of yellow fever after test injections, and their serum failed to protect. The mice inoculated with the same mixtures survived, except 1 in each of 2 sets.

From this evidence it is apparent that the virus in a filtered 10 per cent suspension of fixed virus in immune serum was not neutralized in 4 hours at 28°C. but that it was entirely neutralized and inert after 24 hours at 28° or 3°C.

Experiment B.—A filtered 10 per cent suspension of fixed yellow fever virus in normal monkey serum was divided into two parts. To one was added an equal part of normal monkey serum and to the other an equal part of immune monkey serum. In testing these mixtures in monkeys, 0.06 cc. per kilo of weight was injected subcutaneously. Each mixture was placed at 37°C. and tested immediately and after 0.5, 2, and 6 hours. All the mixtures without immune serum, and also those which contained immune serum but which had been kept at 37°C. for 2 hours or less, produced immunity in monkeys and killed nearly all the mice inoculated intracerebrally. But the mixture of immune serum and fixed virus, which had been kept at 37°C. for 6 hours, permitted all but 1 of the 6 mice inoculated to survive. Two monkeys injected with the same mixture survived test inoculation, but the titres of their sera were low (1/32 and 1/64), while the titres of the sera of the other monkeys in the experiment were about 1/256. One of the 2 monkeys showed the typical leucopenia and the other did not. It appears that the immune serum in this experiment had not neutralized the virus in 2 hours but had neutralized it almost, but not quite, completely in 6 hours.

Experiment C.—In a third experiment the same basic 10 per cent suspension of fixed virus in normal serum was used as in Experiment B, and equal volumes of varying dilutions of the immune monkey serum used in that experiment were added. The final concentrations of immune serum in the mixtures were 1/2, 1/8, 1/32, 1/128, 1/512, and 0. The mixtures were kept at 37°C. for 48 hours to permit maximum neutralization, and then each was injected intracerebrally into 6 mice. Mixtures with immune serum concentrations of 1/32 or over permitted all, or all but 1, of the mice in each set of 6 to survive, but the mixtures with concentrations of 1/128 or less, caused all, or all but 1, of the mice to die. It follows that the minimum concentration of the immune serum which would neutralize the 5 per cent content of fixed virus, minus the loss by filtration, in 48 hours at 37°C., lay between 1/32 and 1/128.

From these experiments the following conclusions are drawn.

(*a*) Neutralization of yellow fever virus by immune serum takes place very slowly at room temperature. It must have been far from complete in the mixtures of immune serum and virus used by us in immunizing man and monkey, and it must have ceased when the mixtures were dried.

(*b*) When yellow fever virus is completely neutralized by immune serum *in vitro*, it loses its power to immunize or infect.

(*c*) The injection of a mixture of yellow fever virus and immune serum, under the conditions of our experiments, is approximately equivalent to the simultaneous injection of the two components.

The observation that the degree of immunity produced by vaccination was almost as high as that following the naturally acquired disease was contrary to expectation, as it had seemed probable that an amount of immune serum capable of inhibiting symptoms would also limit the immunizing reaction. To find out whether extreme quantities of serum would prevent or diminish the production of immunity the following experiment was performed.

A monkey was inoculated subcutaneously with 6 cc. of immune monkey serum per kilo of body weight and 1 hour later it was inoculated subcutaneously with 0.06 cc. (per kilo) of the same 5 per cent filtered suspension of fixed virus in normal monkey serum as was used in Experiment B. The amount of immune serum was 20 times the usual amount used in vaccination and corresponded to 420 cc. for a man weighing 70 kilos. 14 days after the injections, when passive immunity was still present, the protective power of the monkey's serum was low (titre, 1/16), and 62 days after the injections the serum protected only when tested in full strength (titre, 1/1). Daily leucocyte counts showed that the usual leucopenia did not occur. Elevations of temperature were observed and attributed to the excessive amount of serum injected. A test inoculation of virus of the Asibi strain was given 73 days after the injections, when some passive immunity was probably present. The monkey survived.

The injection of extremely large amounts of immune serum may greatly diminish, and perhaps prevent, the production of protective antibodies in the blood after injections of yellow fever virus.

The minimal amount of immune serum, per kilo of body weight, which will protect monkeys against the highly virulent Asibi strain of yellow fever virus appears, from our experience, to be also the minimal amount needed for protection of men or monkeys against the fixed strain having low virulence for them.

SUMMARY AND CONCLUSIONS

1. After preliminary experiments in monkeys, 15 persons were actively immunized by a single injection of a dried mixture of living yellow fever virus, fixed for mice, and human immune serum, with separate injections of enough additional serum to make up the amount required for protection.

2. One person was similarly immunized by injecting immune serum and dried virus separately.

3. By titration of the sera of vaccinated persons in mice, it was shown that the immunity rose in a few weeks to a height comparable to that reached after an attack of yellow fever, and remained there throughout an observation period of 6 months.

4. Yellow fever virus could not be recovered from the blood of vaccinated persons or monkeys, except when the latter had received less than the minimal effective amount of immune serum.

5. Neutralization of yellow fever virus by immune serum took place very slowly *in vitro* at room temperature in our experiments, and could not have been an appreciable factor in vaccination with the serum virus mixtures.

6. A mixture of fixed virus and immune serum retained its immunizing power for 8 months when dried in the frozen state and sealed in glass.

7. It appears that the immunizing reaction after yellow fever vaccination was a part of a true infectious process, as was also the observed leucopenia.

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